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14. ABSTRACT

Aberrations in expression of cyclin E, a positive cell cycle regulator at the G1 to S transition, may affect the biological behavior of breast cancer. In a retrospective analysis of tumor specimens from 395 breast cancer patients, we found that overexpression of the full-length cyclin E protein and its LMW isoforms was a very strong predictor of breast cancer death regardless of whether patients had lymph node-negative disease or lymph node-positive disease ($p < 0.0001$). Our hypothesis is that dysregulation of cyclin E is a powerful prognostic indicator of outcome in early stage breast cancer. Specifically we will: 1: use cyclin E antibody as a prognostic marker for stage I and II breast cancer in a PROSPECTIVE study, 2: examine the cyclin E associated activity and its immunecomplex formation with key cell cycle regulators in freshly resected tumor samples, and 3: develop an immunohistochemical (IHC) assay for specifically detecting the LMW forms of cyclin E in Breast Cancer. We will correlate the cyclin E alterations in samples with the expression of key cell cycle regulators and clinical biomarkers. If we prospectively confirm cyclin E overexpression correlates with poor outcome, clinicians can more appropriately tailor aggressive systemic treatment to those at greatest risk for systemic metastases

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Prospective study, cell cycle, cyclin E, low molecular weight

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Introduction:

The overall purpose of this 3 year study is to use the altered expression of cyclin E as a prognostic marke for breast cancer.

Cyclin E and breast cancer: Human cyclin E was first identified in 1991 through screening of human cDNA libraries for genes that would substitute for G1 cyclin mutations in yeast [1, 2]. Further studies demonstrated that cyclin E levels were periodic during the cell cycle with levels of protein peaking in G1 [4]. This peak in cyclin E levels also correlated with maximum enzymatic function of the cyclin E-cdk2 complex [4]. The critical role of cyclin E in regulating G1 to S transition was confirmed by 2 studies in the mid-1990s. In one study, microinjection of anti-cyclin E antibodies into fibroblasts during G1 resulted in cell cycle arrest [5]. Conversely, in the other study, constitutive overexpression of cyclin E resulted in shortening of the G1 phase, decrease in cell size, and diminished requirements for growth factors [6].

Many studies point to the relevance of cyclin E alterations in breast cancer. The cyclin E gene is amplified in some breast cancer cell lines [7, 8] and we have shown that this amplification can result in as much as a 64-fold overexpression of cyclin E mRNA that is constitutively expressed across all phases of the cell cycle [9, 10]. Examination of the oncogenic potential of cyclin E in transgenic mice under the control of the bovine ß-lactoglobulin promoter, revealed that lactating mammary glands of the transgenic mice overexpressing cyclin E contained regions of hyperplasia and over 10% of the mice developed mammary carcinomas [11]. Lastly, constitutive overexpression of cyclin E (but not cyclin D1 or A) in both immortalized rat embryo fibroblasts and human breast epithelial cells results in chromosomal instability [12]. Collectively these data provide strong support for the role of cyclin E in breast tumorigenesis.

We believe that the most significant cyclin E alteration is the post-translational cleavage of full length cyclin E into LMW forms that are hyperactive compared to the full-length protein. Some breast cancer cell lines and human breast cancers express up to 5 LMW isoforms of cyclin E

(ranging in size from 34 to 49 kDa), in addition to overexpressing the 50 kDa full-length cyclin E protein [7, 13-15]. These LMW forms are unique to tumor cells and correlate with increasing stage and grade of breast cancer [13, 15-17]. To test the clinical significance of LMW forms of cyclin E in breast cancer prognosis, we measured expression of cyclin E in 395 women with primary breast tumors and correlated cyclin E expression with other established prognostic factors and clinical outcome. Cyclin E levels were the most powerful independent predictor for survival in stage I-III breast cancer [3].

Results

The scope of our Statement of Work for the third and final year of the study was to finish Aim 1: *Aim 1: To use cyclin E antibody as a prognostic marker for stage I and II breast cancer in a PROSPECTIVE study* (months 1-36)

During the year 3 of the study we completed western blot analysis on all the 260 patients with cyclin E and other cell ycle regulators and cloncal endpoints. We are currently performing statistical analysis on this correlation with poor prognsis. This is a continuing analysis and as stated it will be done 5-7 years after diagnosis.

Aim-2 Examine cyclin E associated activity and its immune complex formation with key cell cycle regulators in freshly resected tumor samples.

Our results from in vitro analysis of cyclin E in cell lines [18] suggest that the LMW forms of cyclin E may act as sequestering partners to p27 (or p21) compared to the full-length cyclin E. By binding p27 (or p21) more efficiently than the full-length form, the LMW forms essentially sequester these CKIs from the full-length cyclin E. Furthermore, the LMW forms, despite binding significantly more p27 (or p21) than the full-length form, are resistant to its inhibition. We suggest that similarly in tumor cells with de-regulated cyclin E, the LMW forms of cyclin E act to sequester

p27 (or p21), maintain the activity of the cyclin E/CDK2 kinase complex, contribute towards abrogation of the G1/S checkpoint and thereby provide tumor cells with a growth advantage. To test this hypothesis, we examined the binding of p27 to the LMW forms of cyclin E in tumors from breast cancer patients. Figure 1 shows a representative western blot of 9 tumor tissue lysates, five with low expression of the LMW forms of cyclin E and four with high expression of the LMW isoforms compared to the normal (76N) and tumor (MDA-MB-436) cell line controls. Those tumor lysates that had increased expression of full-length and LMW cyclin E also showed increased expression of p27 overall. Additionally, PCNA levels were increased in those samples with increased expression of cyclin E correlating with a higher proliferative rate. We observed that p27 binds efficiently to all forms of cyclin E (Figure 1B). We next determined activity associated with the cyclin E/CDK2 complex of the tumor lysates (Figure 1C). The tumor lysates that had high expression of the LMW forms showed increased kinase activity, similar to our results from insect cells. Therefore, despite the presence of ample p27 and its efficient binding to the LMW forms of cyclin E, its resistance to p27 ensures an active cyclin E/CDK2 complex.

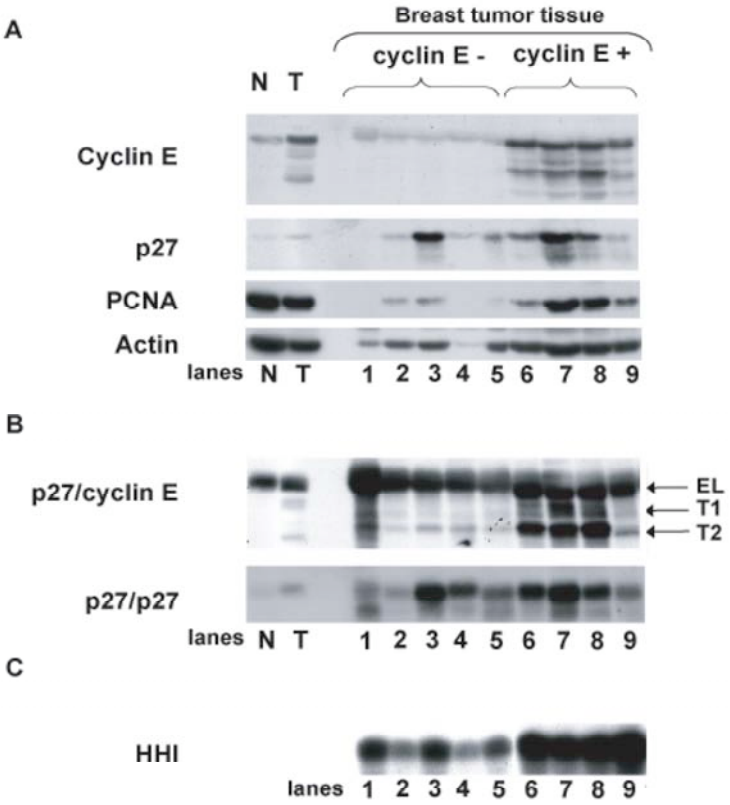


Figure 1. p27 binds the LMW forms of cyclin E, but does not inhibit their kinase activity in breast cancer patient samples. **Panel A:** 25µg of lysate from tumor specimens from patients with normal and elevated levels of cyclin E were used for western blot analysis with the indicated antibodies. The control lanes correspond to cultured 76N normal cell line and MDA-MB-436 tumor cell line. **Panel B:** Immunoprecipitations were performed on 150µg of lysate using polyclonal p27 antibody coupled to protein A beads followed by western blotting with the indicated antibodies. **Panel C:** A kinase assay was performed following immunoprecipitation with polyclonal cyclin E antibody and protein A beads using 150µg of protein extract and histone H1 as substrate.

We have performed similar studies on all the 260 patients and will correlate our findings to survival at the end of the survival analysis 5-7 years post diagnosis as indicated above for aim 1.

Aim 3: To develop Immunohistochemical assay for specifically detecting the LMW forms of cyclin E in breast cancer. We have been successful in completing this aim as well as described below.

Use of cyclin E antibody in immunohistochemistry (Aim1): In aim 3 we proposed to establish cyclin E as a prognostic marker using cyclin E immunohistochemistry (IHC), we would use antibodies that we generated in our laboratory directed to either the EL or the LMW forms of cyclin E. Here we provide evidence for the generation and utility of these cyclin E specific antibodies in IHC.

We have developed a panel of 19 antibodies that target different regions of cyclin E as depicted in Figure 2A. Multi-antigenic peptides were synthesized corresponding to different sequences of cyclin E protein spanning the entire 441 amino acids (Figure 2A). Each antibody was used to detect cyclin EL (full length) and cyclin E-T1 and T2 (the LMW forms of cyclin E) on western blot analysis as well as in immunoprecipitation assays used for kinase assays. The results show that we have already identified antibodies that will specifically detect cyclin E full length (designated antibody ‘r’) and the LMW forms; antibody corresponding to T1 is designated ‘q’ and antibodies labeled ‘a’ and ‘p’ will detect all the LMW forms of cyclin E.

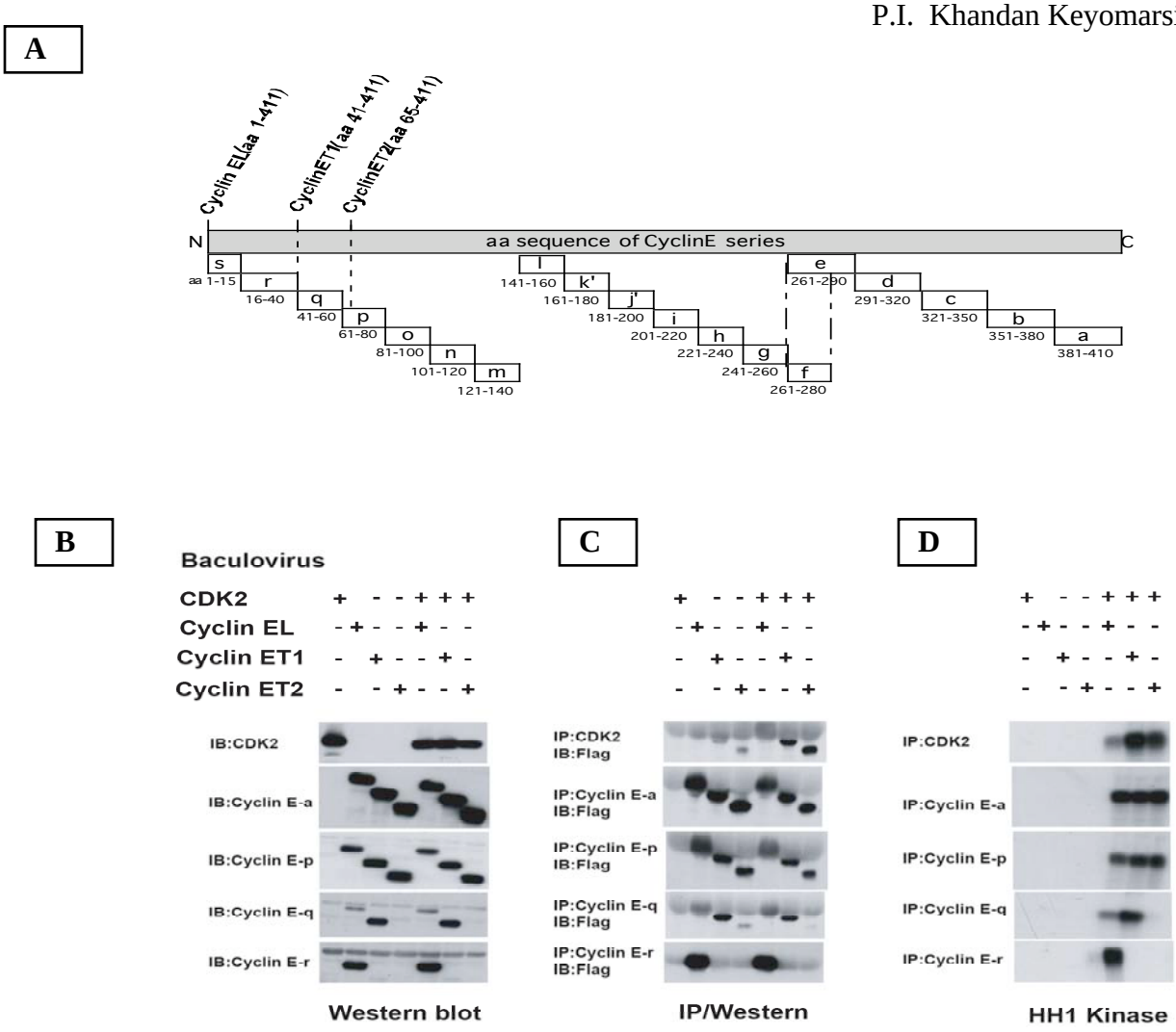


Figure 2: Generation of LMW specific antibodies to cyclin E. Multi-antigenic peptides (between 20 and 30 aa) were synthesized, HPLC purified and used to generate polyclonal antibodies to different regions of cyclin E as depicted in the schematic **(A).** Cell lysates were prepared from insect cells co-infected with baculovirus containing the indicated cyclin E constructs and CDK2. At 60 h post-infection equal amounts of protein were added to each lane (western blot) **(B);** the gel was then subjected to western blot analysis with the indicated antibodies. Immune complex formation (IP/western) with the indicated antibodies were assessed for the same samples by subjecting the different cyclin E specific antibodies immunoprecipitates to western blot analysis using monoclonal antibodies to FLAG **(C)** . Lastly, histone H1 kinase (HH1 kinase) assays were performed on the same cells extracts by IPing equal amounts of cell lysates with the indicated polyclonal antibodies to cyclin E coupled to protein A beads, using histone H1 as substrates **(D).** The autoradiogram of the histone H1 is depicted.

For the examples given in this figure we used insect cell lysates overexpressing each of the full length and the LMW forms of cyclin E (i.e. T1 and T2). These antibodies show specificity for detection of LMW cyclin E using western blot assays (Fig 2B), immunoprecipitation assays (Fig 2C) and kinase assays (Fig 2D).

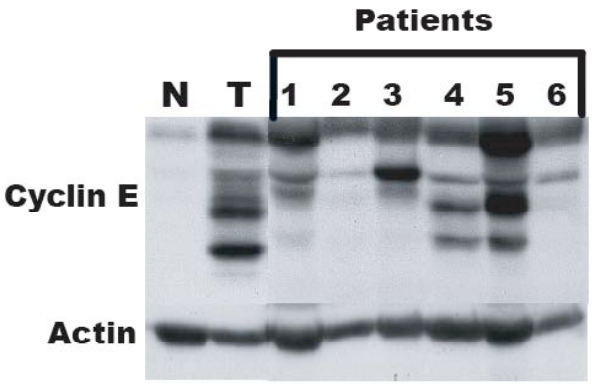
To assess the utility of these antibodies in IHC assays using patient material, we examined the expression of cyclin E (Full length + LMW) both on western blots and IHC using freshly resected breast tissue samples for western blot analysis and paraffin blocks of the same tumors for IHC analysis (Figure 3). The antibody we used for both western blot and IHC analysis is cyclin E-a antibody which can detect total cyclin E (i.e. full length + LMW) forms, since per our previous reports total cyclin E levels have the highest level of significance (i.e. hazard ratio of 13.3) as a prognostic maker in breast cancer [3]. Western blot analysis for cyclin E and Actin (used for equal loading) in tissue samples from 6 patients with breast cancer stage 1 or II is shown in Figure 3A. The protein levels of cyclin E and actin in the western blots were measured by densitometric scanning of the corresponding bands with the use of IP-Lab Gel Software. On the basis of densitometric values the amount of total cyclin E (full length + LMW)/actin were quantitated and presented in Table I.

For the IHC analysis we used formalin fixed, paraffin embedded tissue from the same patients using the same antibody (i.e. cyclin E-a) we used for western blot analysis (Fig 3B). Three different tissue sections from each patient were examined for cyclin E and representative stainings are presented for each patient (Fig 3B). Each slide was scored as percentage of positive area and OD/Pixel and the 2 values were multiplied to quantitate total staining (Table 1). The method of western blot and IHC quantitation is as we previously described [3]. The results from Western and IHC analysis show a very clear concordance between the 2 assays using the cyclin E-a antibody. For example, the IHC and western blot analysis of patient 2 shows no cyclin E staining or expression, while patients 1,3,4,5, and 6 show various degree of cyclin E expression or staining. Of note, is lack of non-specific staining with cyclin E-a antibody, since the slide from patient 2 did not show any staining. This new data shows the successful completion of aim 3.

Figure 3: Cyclin E measurements in tumor tissues from breast cancer patients. (A) Western blots of tumor tissue. Whole-cell lysates were extracted from 6 damples of infiltrating ductal carcinoma. Patients 1,2 and 6 had stage I disease, no pre-operative chemo. Patients 3-5 had stage IIA disease and no pre-operative chemotherapy. Each lane contained 50 µg of protein extract and was incubated with cyclin E-a anitobdy and actin. The control lanes present a cultured normal mammary epithelial cell line, 76N (N) and a cultured breast cancer cell line, MDA-MB-157, (T). **(B)** IHC of the same patients as those analyzed in panel A using cyclin E-a antibody. Parafin embedded tissues were sectioned at 5-µM intervals, placed on coated slides, fixed and stained for cyclin E as previously described [3]. The cyclin E staining for patient #6 was very sporadic and here we are showing representative sections,which express cyclin E and those that did not. **Table 1:** Quantiation of cyclin E expression by western and IHC analysis. For western blot analysis, total cyclin E and actin were measured by densitometric scanning and normalize according to actin densitometric values and the normalized values are shown in the table. The IHC samples were scored form 0-100 on the basis of percent cells stained and 0-1, on the basis of intensity of staining and the 2 number multiplied for total cyclin E staining.

Patients	Western Blot Cyclin E/Actin	IHC %, positive area	IHC OD/Pixel	IHC Total staining
1	1.5	28.6	0.4	11.4
2	.3	1	0.1	0.1
3	1.0	15.4	0.32	4.9
4	1.6	36.8	0.27	9.94
5	2.7	44.3	0.5	22.15
6	.6	8	.27	2.16

A



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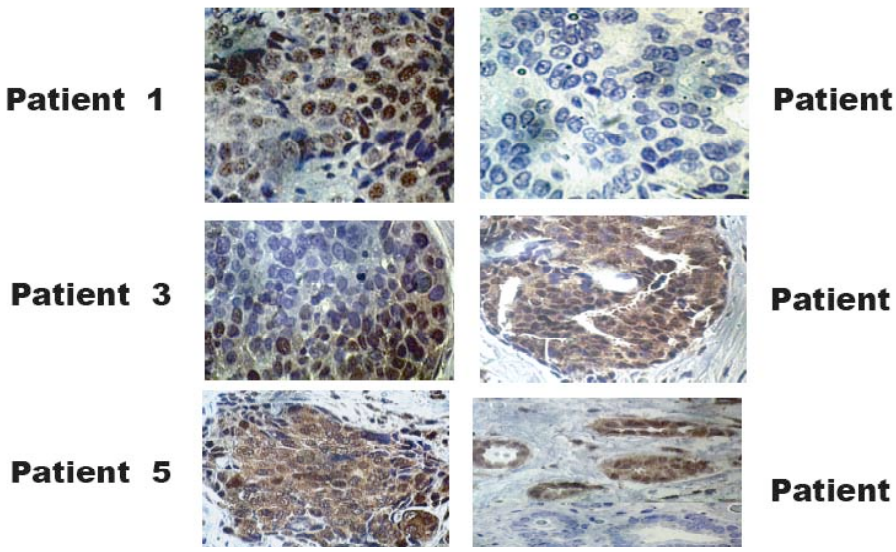

Conclusions/

We have completed all 3 aims of the proposed grant successfully.

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